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# The Modifier Subunit of *Drosophila* Glutamate-Cysteine Ligase Regulates Catalytic Activity by Covalent and Noncovalent Interactions and Influences Glutathione Homeostasis *in Vivo*\*

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Glutamate-cysteine ligase (GCL) has a key influence on glutathione homeostasis. It has been proposed that mammalian GCL is regulated by the redox environment, and we show here that cysteine residues in the *Drosophila melanogaster* GCL modifier subunit (DmGCLM) can form covalent interactions with the catalytic subunit (DmGCLC) and modify its activity. Candidate components of intersubunit disulfides (Cys<sup>213</sup>, Cys<sup>214</sup>, and Cys<sup>267</sup>) were identified using matrix-assisted laser desorption ionization time-of-flight spectroscopy of iodoacetamide-modified DmGCLM as well as examination of the evolutionary conservation of cysteines. Mutation of the 3 cysteine residues allowed DmGCLM to associate with DmGCLC, but inhibited the formation of intersubunit disulfides. This caused a 2-fold reduction in the catalytic efficiency of *Drosophila* GCL, although activity remained significantly higher than the catalytic subunit alone. The cysteine mutant was also more sensitive to inhibition by glutathione than the unmodified holoenzyme. Notably, human GCLM could substitute for DmGCLM in modification of DmGCLC activity. The role of DmGCLM *in vivo* was examined by analysis of a *Drosophila* mutant (*l(3)Lo580*) containing a P-element insertion in *Gclm*. We found that the P-element is not responsible for the lethal phenotype and separated the recessive lethal mutation from the P-element by recombination. This yielded two fully viable and fertile recombinants bearing the P-element insertion, which Western and Northern blotting indicated is a severely hypomorphic allele of *Gclm*. Glutathione levels were ~2-fold lower in the *Gclm*<sup>Lo580</sup> mutants than in control strains, demonstrating the importance of DmGCLM in the regulation of glutathione homeostasis *in vivo*.

The intracellular redox environment is of critical importance in cell physiology. It has a major influence on signaling pathways and cell fate in response to stress (1). Glutathione is one of the key influences on the redox state of the cell; and accordingly, intracellular glutathione levels are subject to multilateral regulatory mechanisms (2, 3).

A major player in the regulation of glutathione homeostasis

is glutamate-cysteine ligase (GCL),<sup>1</sup> which catalyzes the first and rate-limiting step in *de novo* synthesis of glutathione from precursor amino acids (4). GCL is located in the cytoplasm, and its activity appears to be regulated at several levels by the antioxidant status of the cell. In addition to being subject to transcriptional activation by pro-oxidants (5–7), evidence suggests that the catalytic activity is subject to redox regulation by the reversible formation of disulfide bridges between its two subunits (8, 9). Furthermore, GCL is subject to feedback inhibition by glutathione at concentrations that are physiologically relevant (10). The complex regulation of GCL activity highlights its pivotal role in controlling cellular glutathione synthesis.

GCL is a heterodimer composed of a catalytic subunit (GCLC) and a modifier subunit (GCLM). The presence of GCLM modulates the catalytic properties of GCLC by lowering its sensitivity to inhibition by glutathione and by increasing its affinity for glutamate (8, 9). The sensitivity of GCLC to inhibition by glutathione is such that it has been proposed that GCLC would function poorly *in vivo* without the presence of GCLM. The pioneering biochemical studies of Meister and co-workers (9) in the 1990s provided evidence to suggest that GCLM could further enhance the function of GCLC by the formation of intersubunit disulfide bonds. This proposition was based on the observations that treatment of the GCL holoenzyme with dithiothreitol (DTT) lowered its affinity for glutamate and increased its sensitivity to inhibition by the glutathione analog ophthalmic acid. The effects of DTT were dependent on the presence of GCLM. The results prompted the hypothesis that intracellular GCL activity could be increased under conditions that deplete glutathione, where the oxidizing environment within the cell would promote disulfide bond formation within GCL.

More recently, the role of intermolecular disulfide linkages in modifying GCL activity was investigated by mutagenesis of cysteine residues in the catalytic subunit (11). 8 of the 14 cysteine residues in human GCLC were singly altered to glycine, and the effects on activity and ability to form disulfide linkages were examined. One of the 8 cysteines (Cys<sup>553</sup>) was shown to be involved in influencing the ability of GCLM to increase the activity of GCLC. The mutant holoenzyme was, however, still able to form a 114-kDa complex when analyzed by SDS-PAGE under non-reducing conditions. This suggests

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<sup>1</sup> The abbreviations used are: GCL, glutamate-cysteine ligase; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

TABLE I  
Sequences of oligonucleotides used for mutation of DmGCLM cysteine residues

| Mutant   | Oligonucleotide sequence <sup>a</sup>  | Cysteine location  |
|----------|--|--------------------|
| DmGCLM-A | 5'-CAACCTGTCTACGTcCTGCGTGGTGCCAC-3'    | Cys <sup>213</sup> |
| DmGCLM-B | 5'-CAACCTGTCTACGTGCTcCTGCGTGGTGCCAC-3' | Cys <sup>214</sup> |
| DmGCLM-C | 5'-GCAGGAGTTcTCCACCGCTACCC-3'          | Cys <sup>224</sup> |
| DmGCLM-D | 5'-GTGCATGTcCGCTCCGGGGCG-3'            | Cys <sup>267</sup> |
| DmGCLM-E | 5'-CCAAACCGCCCTCTcCGAGGATTCC-3'        | Cys <sup>139</sup> |

<sup>a</sup> The mutated nucleotides are in lowercase letters.

that covalent interactions could still occur between the mutated catalytic subunit and GCLM, indicating that Cys<sup>553</sup> may not be the only cysteine involved in GCLC association with GCLM. It is possible that more than one disulfide linkage may form between the subunits.

We reasoned that the most straightforward method of investigating the importance of intermolecular disulfide linkages in GCL catalysis would be to target the cysteine residues in the modifier subunit; GCLM has fewer cysteines, and interpretation of data would not be confounded by mutation of cysteine residues with a role in catalysis (12). We have shown previously that GCL from *Drosophila melanogaster* contains an ~31-kDa modifier subunit (DmGCLM) that is capable of forming disulfide linkages with the ~80-kDa catalytic subunit (DmGCLC) *in vitro* (13). The disulfide status of specific cysteines in either DmGCLC or DmGCLM is unknown. In this study, we have investigated the importance of intermolecular disulfide linkages in DmGCL activity and show that the ability to form disulfide bonds impinges on both activity and sensitivity to feedback inhibition by glutathione. We also show that *Drosophila Gclm* mutants, although viable, have approximately half the levels of glutathione compared with the control strains, demonstrating the importance of DmGCLM in glutathione homeostasis *in vivo*.

#### EXPERIMENTAL PROCEDURES

**Construction of pET20bDmGCLC**—To make recombinant DmGCLC with a histidine tag at the C terminus, the open reading frame of the *Gclc* gene was excised from pETDmGCLC (13) and subcloned into the NdeI and XhoI sites of pET20b (Novagen), generating pET20bDmGCLC. A 254-bp fragment from the 3'-end of the open reading frame was amplified from pDmGCS4.3.3 (14) by PCR using upstream (5'-GGGAATTCGCCGGCAGCTAATCACCACG-3') and downstream (5'-CCGCTCGAGTTTCTCTCGCAGCAGCC-3') oligonucleotides designed to insert an EcoRI site into the 5'-end and an XhoI site into the 3'-end of the fragment and to remove the terminal stop codon. The 254-bp cDNA fragment was subcloned into the EcoRI and XhoI sites of pBluescript SK<sup>-</sup> and sequenced before it was digested with SgrAI (underlined sequence) and XhoI and used to replace the corresponding SgrAI/XhoI fragment in pET20bDmGCLC.

**Site-directed Mutagenesis**—Site-directed mutagenesis was used to alter the cysteine residues within DmGCLM and was carried out using the QuikChange site-directed mutagenesis kit (Stratagene). Mutations were introduced via PCR-based site-directed mutagenesis using the DmGCLM open reading frame cloned into pBluescript SK<sup>-</sup> (13) as a template and pairs of complementary oligonucleotides (Table I). Oligonucleotides were designed to introduce a single nucleotide change resulting in an amino acid substitution from cysteine to serine. Mutations were introduced sequentially. Amplified plasmid DNA was isolated, and the presence of point mutation(s) was determined by sequencing before the mutated open reading frame was subcloned into the NdeI and XhoI sites of pET15b (Novagen) to generate pETDmGCLM plasmids, which were used to express mutant recombinant DmGCLM polypeptides.

**Expression and Purification of Recombinant Proteins**—pET20bDmGCLC and mutant forms of pETDmGCLM were expressed separately in *Escherichia coli* strain BL21(DE3) and purified by nickel-agarose chromatography as described previously (13), except that LB broth was used in place of Terrific Broth for the culture medium. Recombinant human GCLM (HsGCLM) cloned into pET15b was expressed in BL21(DE3) cells and purified as described previously (15). Preparations of the GCL holoenzyme were generated by mixing purified recombinant DmGCLC (with a C-termi-

nal histidine tag) and GCLM polypeptides (each tagged with histidine at the N terminus) and purifying the protein complexes by gel filtration chromatography under the conditions described previously (13).

**Carboxymethylation of Cysteine Residues within DmGCLM**—Samples of DmGCLM were diluted to a final concentration of 200 µg/ml with 100 mM Tris-HCl (pH 8.0) and 1 mM EDTA, containing no additives, 5 mM DTT, or 5 mM DTT plus 6 M guanidine HCl. Samples were incubated with 10 mM iodoacetamide for 5 h in the dark at room temperature and were subsequently dialyzed overnight in 100 mM Tris-HCl (pH 8.0) and 1 mM EDTA in the dark (16). The dialyzed samples were digested with trypsin and analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy as described (17) and performed by Douglas J. Lamont at the "FingerPrints" Proteomics Facility, Post-Genomics and Molecular Interactions Centre, School of Life Sciences, University of Dundee.

**Circular Dichroism Spectroscopy**—Samples of mutant and unmodified DmGCLM polypeptides (1 mg/ml) for CD analyses were dialyzed overnight in 20 mM Tris-HCl (pH 7.4) and degassed by bubbling N<sub>2</sub> through the samples. The CD analyses were carried out using the CONTIN procedure (18) by Dr. Sharon Kelly at the Circular Dichroism Facility of the University of Glasgow (Glasgow, United Kingdom).

**Analysis of GCL Activity**—GCL activity was determined at 25 °C spectrophotometrically (13) using L-α-aminobutyric acid as a substrate instead of L-cysteine and adapted for use on 96-well plates. Reaction mixtures (0.21 ml) contained 5 mM L-glutamate, 50 mM L-α-aminobutyric acid, 2.4 mM phosphoenolpyruvate, 0.24 mM NADH, 1 unit of lactic dehydrogenase, and 1 unit of pyruvate kinase. The reaction was initiated by the addition of ATP to a final concentration of 5 mM. L-Glutamate concentrations ranging from 0.25 to 16 mM were used to determine the *K<sub>m</sub>* and *V<sub>max</sub>* values from initial reaction rates. Michaelis-Menten parameters were fitted via a Hanes plot using hyperbolic regression analysis software (19). For inhibition studies with glutathione, reduced glutathione was included in the standard reaction mixture at concentrations between 0.25 and 16 mM.

**Western Blot Analysis**—Western blotting was performed using anti-serum raised against recombinant DmGCLM, DmGCLC, or HsGCLM as described previously (13, 15). For analysis of DmGCL content in flies, 10 male flies were homogenized in 150 µl of sample loading buffer (100 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 3.6 M 2-mercaptoethanol, and 0.05% (w/v) bromophenol blue) and incubated at 100 °C for 5 min before being subjected to SDS-PAGE and Western blotting.

**Glutathione Determination**—The total glutathione content of whole flies was determined by a modification of the glutathione reductase/5,5'-dithiobis(2-nitrobenzoic acid) recycling assay described by Tietze (20) and adapted for use on a 96-well plate. Routinely, 10 male flies were manually homogenized in 100 µl of ice-cold 10% (w/v) sulfosalicylic acid. Reaction mixtures (0.15 ml) contained 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) and 0.34 mM NADPH in 150 mM sodium phosphate buffer (pH 7.5) containing 7.5 mM EDTA. The reaction was initiated by the addition of 0.1 unit of glutathione reductase (Sigma). Glutathione concentrations were determined from a standard curve generated from known concentrations of glutathione prepared in 10% (w/v) sulfosalicylic acid and are expressed as picomoles of glutathione/fly. To determine whether glutathione levels differed significantly by genotype, we used a one-way analysis of variance with *post hoc* analyses carried out using Scheffé's test and the SPSS statistical software package. Values were considered to be significantly different from each other when *p* < 0.05.

**Drosophila Procedures**—Flies were maintained on a standard oatmeal medium. We obtained the P-element insertion stock *l(3)LO580* from the Bloomington *Drosophila* Stock Center. This stock is reported to bear a recessive lethal mutation insertion of a *P[acW]*-element (21) within the 5'-untranslated region of *Gclm*. The location of the P-element was confirmed by PCR and sequencing. The recessive lethal present on the *l(3)LO580* chromosome was removed by recombination with an *e<sup>4</sup> wo<sup>1</sup> ro<sup>1</sup>* stock obtained from the Bloomington *Drosophila*



TABLE II  
Cysteine-containing tryptic fragments of DmGCLM

| Residues | Fragment   |
|----------|--|
| 137-147  | PPC <b>E</b> SEDNSVSR  |
| 177-260  | QQITQLGIADLDAAALEELHNSAQVVPTIAQVNLST <b>CC</b> VVPPELQ <b>EFC</b> TAHDIQLNTHSDPEL<br>LLPVEQFDGLVPGYTIDWTLR |
| 267-268  | <b>D</b><br><b>CR</b>  |

Stock Center. Recombinant progeny bearing the *L0580* P-element insertion (marked with *w*<sup>+</sup>) and either *e*<sup>4</sup> or *ro*<sup>1</sup> were selected and tested for recessive lethality. Approximately 50% of the recombinants of each class were homozygous viable. We did not map the location of the lethal mutation. Viable stocks bearing the *L0580* P-element and marked with either *e*<sup>4</sup> or *ro*<sup>1</sup> were established. We have named the *L0580* P-element insertion *Gclm*<sup>*L0580*</sup> to indicate its identity as a mutant allele of *Gclm*. Revertants were obtained from the *e*<sup>4</sup> *Gclm*<sup>*L0580*</sup> stock by crossing to a stock bearing a third chromosome insertion of the P{ry<sup>+</sup>Δ2-3}-element, which produces constitutively active P-transposase. Revertants were selected on the basis of loss of eye pigmentation, associated with loss of the *w*<sup>+</sup>-bearing P-element. We confirmed the loss of the element by PCR. No apparent aberrations were seen. The level of DmGCLM in the revertant stock was estimated by Western blotting.

**Multiple Sequence Alignment**—Multiple sequence alignments were performed using the Genetics Computer Group Pileup software.

## RESULTS

**Mutation of All 5 Cysteines within DmGCLM Inhibits Interaction with DmGCLC**—DmGCLM contains 5 cysteine residues at positions 139, 213, 214, 224, and 269, which were named E, A, B, C, and D, respectively (Table II). To investigate the role of intersubunit disulfide linkages in the regulation of DmGCL activity, we performed site-directed mutagenesis on the modifier subunit to generate a polypeptide that entirely lacks cysteine (DmGCLM-FM). Purified recombinant DmGCLM-FM appeared as a single polypeptide with an apparent molecular mass of ~35 kDa when analyzed by SDS-PAGE (Fig. 1). Its mobility is slower than that of wild-type DmGCLM (Fig. 1), which has an apparent molecular mass of 31 kDa (13). The reason for the difference in electrophoretic motility is unknown, but it suggests that DmGCLM-FM may have a less compact structure than the wild-type protein. The secondary structures of the polypeptides were examined by CD spectroscopy over the absorbance range 260 to 320 nm. The CD analyses did not, however, reveal any significant differences between DmGCLM-FM and the unmodified DmGCLM polypeptide (data not shown). The predicted composition of α-helices and β-sheets in DmGCLM is 6 ± 1.3 and 61 ± 1.45%, respectively, similar to that of DmGCLM-FM (7 ± 1.5 and 62 ± 1.6%, respectively).

Regulation of mammalian GCLC activity by GCLM is thought to involve, at least in part, the transient formation of reversible covalent interactions promoting conformational change(s) around the active site of GCLC (9). To investigate the role of these interactions in DmGCL regulation, we attempted to generate a DmGCL holoenzyme using DmGCLC and DmGCLM-FM.

DmGCLM-FM was mixed with DmGCLC, and the mixture was subjected to gel filtration chromatography. When the wild-type DmGCLC/DmGCLM mixture was resolved, two peaks

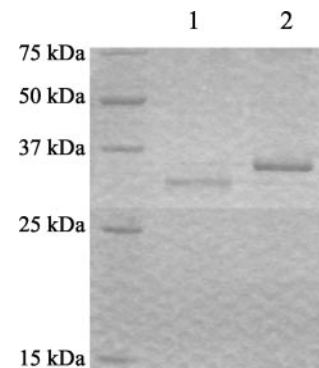
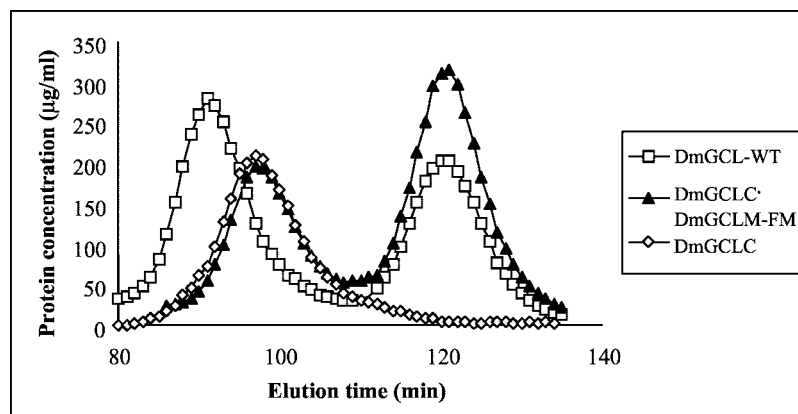


FIG. 1. **SDS-PAGE analysis of DmGCLM-FM.** Recombinant DmGCLM polypeptides were purified from *E. coli* by nickel-agarose chromatography and analyzed by SDS-PAGE. Protein (0.75 μg) was loaded as follows: lane 1, DmGCLM; lane 2, DmGCLM-FM.

were identified corresponding to the holoenzyme complex (~140 kDa) and uncomplexed DmGCLM (~30 kDa) (Fig. 2) (13). However, when the DmGCLC/DmGCLM-FM mixture was resolved, a major peak corresponding to the ~140-kDa complex was not observed, and the profile obtained was more akin to that of uncomplexed DmGCLC (~80 kDa) and DmGCLM (~30 kDa) (Fig. 2). This was confirmed by SDS-PAGE analysis of the peak fractions obtained from the DmGCLC/DmGCLM-FM profile, which showed that DmGCLM did not co-elute with DmGCLC in the ~80-kDa peak (data not shown). A small shoulder on the leading edge of the 80-kDa peak was observed and found to contain a trace of DmGCLM. Analyses of these fractions for GCL activity did not demonstrate an enhancement of activity compared with the catalytic subunit alone (data not shown). These findings suggest that mutation of all of the cysteine residues in DmGCLM substantially impairs its ability to interact with DmGCLC.

**Redox Status of Cysteine Residues in DmGCLM**—The disulfide status of cysteine residues in DmGCLM was analyzed by peptide mass fingerprinting. Recombinant DmGCLM was treated with iodoacetamide under native, reducing, or reducing and denaturing conditions. Tryptic digests of iodoacetamide-treated DmGCLM were analyzed by MALDI-TOF mass spectrometry. The iodoacetamide-modified cysteine-containing polypeptide fragments are shown in Table II. In native DmGCLM, the fragment containing Cys<sup>139</sup> (residues 137–147) and the fragment containing Cys<sup>213</sup>, Cys<sup>214</sup>, and Cys<sup>224</sup> (residues 177–260) were both singly modified by iodoacetamide (data not shown). In the reduced sample, a further iodoacetamide modification was identified in the fragment containing Cys<sup>213</sup>, Cys<sup>214</sup>, and Cys<sup>224</sup>, whereas under denaturing and reducing

**FIG. 2. Gel filtration of DmGCLC combined with DmGCLM-FM.** Purified recombinant DmGCLM-FM (10 mg) was mixed with purified DmGCLC (10 mg). Uncomplexed DmGCLC ( $\diamond$ ), wild-type (WT) DmGCL ( $\square$ ), and the DmGCLC/DmGCLM-FM combination ( $\blacktriangle$ ) were separately subjected to gel filtration chromatography as described under "Experimental Procedures." Eluted fractions were analyzed for protein concentration.



HGCLM 1 MGDSTRAAKALLARARTLHLOTGNL...LNWGRLLKKCPSTSEELHDCIOKTLNEWSSQ  
 XGCLM 1 MGDSTAAARTLLDKADTLHLOTGNL...LNWGLKKKCPSTSEELQDCIRTTINEWNQK  
 ZGCLM 1 ~MEHHVNAKRLFSHATTKLHLOTGNL...VNRSLKKKCPSTSEELQDCIOGTLSEWFTA  
 CeGCLM 1 ~MYCHQFLNLSLTLRFRHTGNV...NGYTLKVRFRFETSAEELAACDLQLS...SNP  
 DmGCLM 1 ~~~~~~MIPTTKYQNVVISTGNI...LATELGQK...SNEELYDGLKTHHTDSTA  
 SpGCLM 1 ~~~~~~MONEKKHLLYTGDTVKN...KSGIWNASRIKENLELWKALENVRLTKFTGD

HGCLM 58 INDLVREFFPV...LECTVSHAVEKINPDEREEMKVS AKLFIVESSSSSTSAVDMAC  
 XGCLM 58 ISPFLVKIISQT...LECTVSCAMETINLPDEREEMKVS VKLFIV...SPSSSSVTBAIDMAC  
 ZGCLM 57 MPSSIDSEIPSV...LDCSTPENTPATPDEREELKVS VKLFLTEW...DCSSIRSAVDMAC  
 CeGCLM 55 IADF.KENSCL...LLCDNDH...IDNYERDEIKITMKVFLSAW.DVQCIDCAVTSLK  
 DmGCLM 48 ERVVVEKEIDBLHGRVQRATQELTTRITENGRNETSIGAKFINR.HSTBSVNCABELL  
 SpGCLM 52 GDENIKKNIKVLVP...VNEKPQKID.GKQEFYEITVKLFELLEGEND.IKKREBTL

HGCLM 115 SMLGVAQIDSVIIA.....SPPLE...DGVNLS.....LEHLQPY  
 XGCLM 114 STLGVACIDSVIIA.....PPPLE...DGRSVS.....LENLQPY  
 ZGCLM 113 LSLGVQIDSVFIA.....PPPLP...EGEAQ.....LTHLQPY  
 CeGCLM 106 EQIKTKDIEVILS.....RPELILIDGESEED...ENNRWFEKVKPL  
 DmGCLM 107 HILSVTHVDNVVLAHPNAVATATPVATTKPFCESDSNVSRATNWSQRNGKEGVAELKEL  
 SpGCLM 105 QVFYNLHM...LFEIDFVSTLVVSFPHTIFLKEGSGNSSSNEIYDSIDEIPPQELQSVWDT

HGCLM 147 WEELERLVQSKKIVAIGTSDLDKTLQELYQWAQVKPNSNQVNLASCCVMPDPLTAFAKQ  
 XGCLM 146 WEELERLVQDAKVVISIGTSDLDKALQELYLWSQVKPASNQVNLASCCVMPDPLTEFAKQ  
 ZGCLM 145 WOELERLVQSKKIVAIGTSDLDKTLQELYNWAQTKPSSNQVNLASCCVMPDPLTAFAKE  
 CeGCLM 146 YTYMEKLVSEIASIGVSDFSARQKTLIDHFDVVKPSINHVRLDCCCVPPPELQALAND  
 DmGCLM 167 YKTLQYALKQCIQGLGADLAAALEELHNSAQVVPVIAQVNLSTCCVPPPELQEBCTA  
 SpGCLM 162 WKILKEKVGEKIGTLGVSEFGVNELERLISSVNVVVERSTQINIGQNKIPNDILNFADR

HGCLM 207 FDIQLLTHNDPKELLSEASFQ...EALQESIPDIQAHEWVPIWLLRYSVIVKSRGIKSK  
 XGCLM 206 FDIQLLTHNDPKELLSEEDFC...EALKESVPECHFNWSPWILKYSVIVKTRGIKIK  
 ZGCLM 205 FDIQLLTHSDPKELISAAGFQ...EAVQGSQETQVDDWRLEWVLRYSIVKSRGIKAK  
 CeGCLM 206 NDVQLLVHNDTPFPNNIFKTFCEIDSGSKAKVCSPLFETTWLSRYSVWVRKRSINTSK  
 DmGCLM 227 HDIQLNTHSDEELLPEVEQFDGL.....VPGYTTDWTLYQVHVRCRGVITAK  
 SpGCLM 222 HHKLFHHSDEFSALLSESE...ITSVTHKCPENPNA.RVDWVTRYTITRHTAVIHOK

HGCLM 264 GYILQAKRRGS~  
 XGCLM 263 GYILQAKRKGSF  
 ZGCLM 262 GYIVHAKKSNH  
 CeGCLM 266 GYIVQFFRKHD~  
 DmGCLM 275 GYIVGASRSSV~  
 SpGCLM 278 GYIVQSTYTE~

conditions, all 3 cysteine residues within this fragment were modified. We were unable to identify the fragment containing Cys<sup>267</sup> (residues 267–268) due to its being small and poorly retained on the high pressure liquid chromatography column. Nevertheless, MALDI-TOF analysis implicated Cys<sup>267</sup> as being involved in disulfide formation, as peaks corresponding to mixed disulfides between Cys<sup>267</sup> and Cys<sup>139</sup> and between Cys<sup>267</sup> and Cys<sup>213</sup>, Cys<sup>214</sup>, or Cys<sup>224</sup> were identified when the non-reduced iodoacetamide-treated samples were analyzed. These peaks were absent in samples treated with DTT.

It is important to note that, in the absence of DmGCLC and

glutathione or DTT, purified recombinant DmGCLM was predominantly multimeric (data not shown). This suggests that some of the disulfide bonds that become reduced upon DTT treatment and subsequently modified by iodoacetamide are likely to be intermolecular disulfides rather than intramolecular disulfides. It is unclear whether this has biological significance, but it is possible that multimerization may occur as a result of the artificial environment of the *E. coli* expression system, in which reactive cysteines could readily form intermolecular disulfide bonds giving rise to homodimers or trimers. We reasoned that cysteine residues involved in forming inter-

**FIG. 3. Alignment of GCLM amino acid sequences from different phyla and animal classes.** Black boxes show amino acid sequence identity. Gray boxes show amino acid similarity. The conserved cysteine residues potentially involved in redox regulation of GCL activity are indicated by asterisks. *Xl*, *X. laevis*; *Dr*, *D. rerio*; *Ce*, *C. elegans*; *Sp*, *S. pombe*.

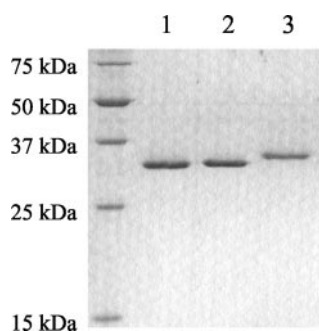


FIG. 4. SDS-PAGE analysis of DmGCLM-ABD and DmGCLM-CE mutants. Recombinant DmGCLM polypeptides were purified from *E. coli* by nickel-agarose chromatography and analyzed by SDS-PAGE. Protein (0.75  $\mu$ g) was loaded as follows: lane 1, DmGCLM; lane 2, DmGCLM-ABD; lane 3, DmGCLM-CE.

molecular disulfide bonds in the absence of DmGCLC are potential candidates for forming disulfide bridges with DmGCLC in the holoenzyme.

Our findings from the proteomic analyses indicate that two cysteines on the surface of DmGCLM are predominantly in a reduced state and can be modified by iodoacetamide, two surface cysteines are involved in formation of disulfide bonds, and one cysteine is inaccessible to iodoacetamide without denaturation of the protein. The MALDI-TOF analyses suggest that Cys<sup>139</sup> is principally (but not entirely) present as a free thiol on the surface of DmGCLM and that Cys<sup>267</sup> can participate in disulfide bridge formation with other cysteines. Tryptic fragment 177–260 appears to contain one free thiol, one cysteine as part of a disulfide, and one cysteine that is buried within the native protein. A caveat to our interpretation is that the vicinal cysteines may not be amenable to simultaneous modification by iodoacetamide in the nondenatured protein. Disulfide shuffling may also occur. Due to the positions of the tryptic cleavage sites, we were unable to dissect this further, and we used a bioinformatics approach to gain additional insight into which of the cysteine residues in tryptic fragment 177–260 could be involved in disulfide formation with DmGCLC.

Comparison of the amino acid sequences of DmGCLM, HsGCLM, and hypothetical GCLM polypeptides from *Caenorhabditis elegans* (GenBank<sup>TM</sup>/EBI accession number NP\_491305), *Xenopus laevis* (accession number AAH44107), and *Danio rerio* (accession number AAH44532) showed that both Cys<sup>213</sup> and Cys<sup>214</sup> are conserved in representatives from mammals, amphibians, fish, arthropods, and nematodes (Fig. 3). A partial sequence of an expressed sequence tag encoding a hypothetical GCLM cDNA from *Gallus gallus* (accession number AJ447023) also contained the conserved Cys<sup>213</sup> and Cys<sup>214</sup> residues (data not shown). The only putative GCLM sequence that we found that does not contain both of the conserved cysteines was from *Schizosaccharomyces pombe* (accession number NP\_588368), in which only Cys<sup>214</sup> is conserved (Fig. 3). Assuming that the sole function of GCLM is to regulate GCLC activity and that intermolecular disulfide bridges are involved in the regulation of activity *in vivo*, the evolutionary conservation of cysteine residues infers a possible role for Cys<sup>213</sup> and/or Cys<sup>214</sup> (rather than Cys<sup>224</sup>) in the regulation of DmGCLC activity.

We went on to generate a mutant form of DmGCLM lacking Cys<sup>213</sup>, Cys<sup>214</sup>, and Cys<sup>267</sup> (named DmGCLM-ABD). We also generated the converse mutant lacking Cys<sup>139</sup> and Cys<sup>224</sup> (named DmGCLM-CE) and investigated the ability of both mutants to form the 140-kDa holoenzyme complex.

**Mutation of Cys<sup>213</sup>, Cys<sup>214</sup>, and Cys<sup>267</sup> Impairs Intermolecular Disulfide Bridge Formation within DmGCL**—Purified recombinant DmGCLM-ABD appeared as single polypeptide with an approximate molecular mass of 31 kDa when analyzed

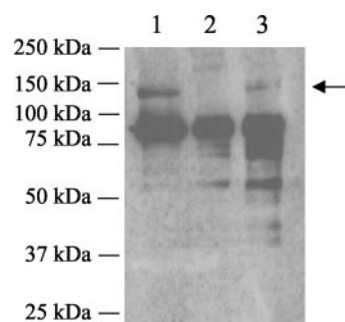


FIG. 5. Analysis of intermolecular disulfide bridge formation in the mutant DmGCL holoenzymes. After dialysis to remove DTT, protein (200 ng) from the ~140-kDa peak fractions obtained after gel filtration chromatography of DmGCLC/DmGCLM (lane 1), DmGCLC/DmGCLM-ABD (lane 2), or DmGCLC/DmGCLM-CE (lane 3) was resolved by SDS-PAGE in the absence of 2-mercaptoethanol and analyzed by Western blotting using antiserum raised against recombinant DmGCLC.

TABLE III  
Kinetic constants for *Drosophila* GCL with respect to L-glutamate

| Enzyme <sup>a</sup> | $K_m$           | $V_{max}$        | $k_{cat}/K_m$                    |
|---------------------|-----------------|------------------|----------------------------------|
|                     | mM              | $\mu$ mol/min/mg | $\text{min}^{-1} \text{mM}^{-1}$ |
| DmGCL               | $0.91 \pm 0.06$ | $12.1 \pm 0.41$  | $1096.1 \pm 85$                  |
| DmGCLC/DmGCLM-ABD   | $0.97 \pm 0.02$ | $5.85 \pm 0.40$  | $490.46 \pm 33$                  |
| DmGCLC              | $0.92 \pm 0.08$ | $1.51 \pm 0.02$  | $134.44 \pm 11$                  |

<sup>a</sup> Assays were performed in triplicate from one preparation each of DmGCLC, DmGCLM, and DmGCLM-ABD. The catalytic subunit in each preparation is from the same source, and all proteins were purified on the same day under the same conditions. DmGCLC with a hexahistidine tag at the C terminus was used rather than the N-terminal tag employed previously (13).

by SDS-PAGE (Fig. 4). This is in agreement with the molecular mass obtained for wild-type DmGCLM. Purified recombinant DmGCLM-CE also appeared as a single polypeptide, although the estimated molecular mass of ~34 kDa (Fig. 4, lane 3) is similar to that found for DmGCLM-FM. Interestingly, DmGCLM containing a single mutation at Cys<sup>224</sup> also exhibited retarded motility (data not shown). This was not observed in DmGCLM with a single mutation at Cys<sup>139</sup>, implicating Cys<sup>224</sup> in this effect.

DmGCLM-ABD and DmGCLM-CE were each mixed separately with DmGCLC, and the mixtures were resolved by gel filtration chromatography. The profiles obtained following separation of DmGCLC/DmGCLM-ABD and DmGCLC/DmGCLM-CE were identical to that of the wild-type DmGCLC/DmGCLM mixture (data not shown), suggesting that mutation of Cys<sup>139</sup> and Cys<sup>224</sup> together or Cys<sup>267</sup>, Cys<sup>213</sup>, and Cys<sup>214</sup> together has little or no impact on the ability of DmGCLM to associate with DmGCLC to form an ~140-kDa complex. This was confirmed by SDS-PAGE analysis, which showed that the ~140-kDa complexes obtained following gel filtration contained both DmGCLC and the mutant forms of DmGCLM (data not shown).

To explore the role of cysteine residues within DmGCLM in disulfide interactions, we investigated the ability of mutant DmGCLM to form the 140-kDa complex with DmGCLC under non-reducing conditions. The peak fractions obtained from gel filtration were subjected to SDS-PAGE and Western blotting under non-reducing conditions (Fig. 5). In the control DmGCLC/DmGCLM sample, a complex with an approximate molecular mass of 140 kDa was apparent when the sample was probed with antiserum raised against DmGCLC (Fig. 5). A corresponding band was also identified in the DmGCL-CE sample analyzed separately in the same way, suggesting that mutation of Cys<sup>139</sup> and Cys<sup>224</sup> has little impact on disulfide bond



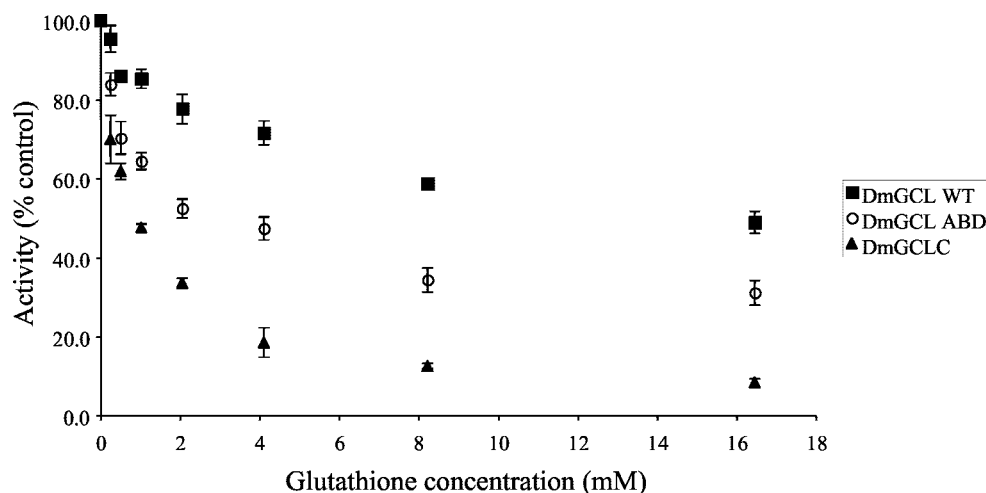


FIG. 6. **Inhibition of recombinant DmGCLC/DmGCLM-ABD activity by glutathione.** The specific activities of the wild-type (WT) DmGCL holoenzyme (■), the DmGCLC/DmGCLM-ABD holoenzyme (*DmGCL ABD*; ○), and DmGCLC (▲) were measured under standard assay conditions in the presence of increasing concentrations of glutathione. Activity is expressed as a percentage of the control activity in the absence of glutathione (mean  $\pm$  S.E.).

formation within DmGCL. Conversely, the 140-kDa band was not detectable in the lane containing DmGCLC/DmGCLM-ABD, suggesting that mutation of these residues inhibits disulfide bond formation between the DmGCL subunits under non-reducing conditions. Taken together with the results from the peptide fingerprinting experiments, these findings implicate Cys<sup>267</sup> and either Cys<sup>213</sup> or Cys<sup>214</sup> (or both) as the cysteine residues within DmGCLM responsible for intermolecular disulfide linkages.

**Cysteine Residues in DmGCLM Impact on GCL Activity—**To determine whether inhibition of intermolecular disulfide bond formation affects the catalytic characteristics of DmGCL, we compared the catalytic activities of the dialyzed DmGCL and DmGCLC/DmGCLM-ABD samples. Kinetic analyses of DmGCL activity were carried out on at least four separate occasions on different preparations of DmGCL. We found that, unlike the  $K_m$  values, which were fairly consistent between experiments, the specific activity and maximal velocity of DmGCLC varied between preparations by as much as 2-fold. Despite this interbatch variation, the magnitude of changes in  $V_{max}$  and  $k_{cat}/K_m$  between the unmodified and mutant DmGCL polypeptides and the catalytic subunit alone was consistent between preparations.

The  $K_m$  of DmGCL for L-glutamate is 0.91 mM, whereas the  $K_m$  of DmGCLC/DmGCLM-ABD is 0.97 mM (Table III). This observation implies that loss of disulfide bond formation has little impact on the affinity of DmGCL for L-glutamate. In contrast, the  $V_{max}$  of DmGCL was found to be 12.1  $\mu$ mol/min/mg, approximately twice that of DmGCLC/DmGCLM-ABD. This suggests that intermolecular disulfide bridge formation has a significant impact on the catalytic efficiency of the DmGCL holoenzyme. The  $V_{max}$  of DmGCLC is substantially lower than that of either DmGCLC/DmGCLM-ABD (~4-fold) or unmodified DmGCL (~8-fold) (Table III), emphasizing the importance of non-covalent subunit interactions in regulating DmGCL activity. In contrast, the  $K_m$  obtained for DmGCLC is not significantly different from that of DmGCL or DmGCLC/DmGCLM-ABD.

**Absence of Intermolecular Disulfide Linkages between DmGCLM and DmGCLC Enhances Sensitivity to Feedback Inhibition by Glutathione—**The DmGCL holoenzyme is subject to feedback inhibition by glutathione (13). Inhibition is mixed, and reduction of intermolecular disulfide linkages by glutathione may facilitate its access to the active site to inhibit activity.

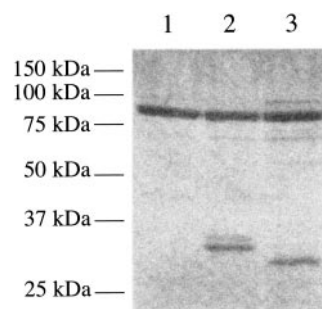


FIG. 7. **Analysis of the subunit composition of the hybrid *Drosophila*/human GCL holoenzyme.** Protein (2  $\mu$ g) from the peak fractions obtained after gel filtration of DmGCLC (lane 1), DmGCLC/DmGCLM (lane 2), or DmGCLC/HsGCLM (lane 3) was resolved by SDS-PAGE in the presence of 2-mercaptoethanol.

We hypothesized that a DmGCL mutant unable to form disulfide linkages would be more susceptible to inhibition by glutathione.

Wild-type DmGCL activity was lowered in the presence of glutathione by a maximum of ~40% (Fig. 6). By contrast, DmGCLC/DmGCLM-ABD activity was more susceptible to inhibition by glutathione; activity was decreased by ~60% at the highest concentration of GSH (16 mM). The difference in the extent of inhibition between DmGCL and DmGCLC/DmGCLM-ABD was particularly evident at lower concentrations of glutathione. These findings highlight the potential importance of intermolecular disulfide bridges within DmGCL in regulating the mechanism of feedback inhibition by glutathione in the holoenzyme. The susceptibility of DmGCLC/DmGCLM-ABD to inhibition by glutathione was not, however, as marked as that of DmGCLC, where activity was almost completely abolished at higher concentrations of GSH (Fig. 6). Inhibition of the catalytic subunit by glutathione is competitive (13), and the difference in the extent of inhibition between DmGCLC and DmGCLC/DmGCLM-ABD underscores the impact that non-covalent intersubunit interactions have upon the susceptibility of DmGCLC to glutathione inhibition.

**HsGCLM Is Able to Interact with DmGCLC in Vitro via Covalent Interactions—**To gain a better understanding about some of the regions on GCLM that may be involved in subunit interactions, we investigated whether we could create a hybrid GCL holoenzyme using HsGCLM and DmGCLC. Purified re-

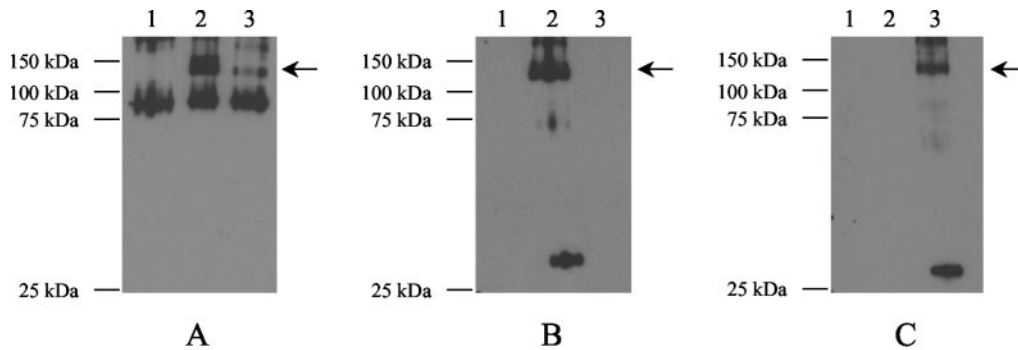


FIG. 8. **Analysis of the subunit behavior of the hybrid *Drosophila*/human GCL holoenzyme under nonreducing conditions.** After dialysis to remove DTT, protein (200 ng) from the peak fractions obtained after gel filtration of DmGCLC (lane 1), DmGCLC/DmGCLM (lane 2), or DmGCLC/HsGCLM (lane 3) was resolved by SDS-PAGE in the absence of 2-mercaptoethanol and analyzed by Western blotting using antiserum raised against DmGCLC (A), DmGCLM (B), or HsGCLM (C).

combinant HsGCLM was mixed with purified DmGCLC and resolved by gel filtration chromatography. A major peak with an estimated molecular mass of ~140 kDa and a minor peak with an estimated molecular mass of ~28 kDa, corresponding to uncomplexed HsGCLM, were identified when the DmGCLC/HsGCLM mixture was separated by gel filtration (data not shown). This was identical to the profile obtained following resolution of the DmGCLC/DmGCLM mixture. Fig. 7 shows that polypeptides corresponding to DmGCLC (~80 kDa) and HsGCLM (~28 kDa) are present in the 140-kDa peak. This indicates that, despite having only 27% sequence identity to DmGCLM, HsGCLM is able to interact with DmGCLC to generate a new higher molecular mass protein complex when they are mixed together *in vitro*.

To determine whether HsGCLM can form covalent interactions with DmGCLC, the ~140-kDa sample was subjected to SDS-PAGE under non-reducing conditions. Western blot analyses using antiserum raised against DmGCLC showed the presence of an ~140-kDa band in the DmGCLC/HsGCLM sample (Fig. 8A), similar to the ~140-kDa band observed when the normal DmGCL holoenzyme was analyzed under the same conditions. This band was absent in the sample containing DmGCLC alone (Fig. 8A). The 140-kDa band was also detected when the DmGCLC/HsGCLM sample was probed with antiserum raised against HsGCLM (Fig. 8C). These results indicate that HsGCLM is capable of forming disulfide linkages with DmGCLC. As only 2 cysteine residues (Cys<sup>213</sup> and Cys<sup>214</sup>) are conserved between the *Drosophila* and human modifier subunits (Fig. 3), it seems likely that either one or both residues are involved in intermolecular disulfide bond formation.

**HsGCLM Can Enhance the Activity of DmGCLC**—The catalytic activity of the wild-type DmGCL holoenzyme was compared with that of the DmGCLC/HsGCLM hybrid. The  $K_m$  for the DmGCLC/HsGCLM hybrid enzyme is 1.01 mM, which is similar to the value of 0.89 mM obtained for the *Drosophila* GCL holoenzyme. The  $V_{max}$  for the DmGCLC/HsGCLM hybrid enzyme was found to be 8.39  $\mu\text{mol}/\text{min}/\text{mg}$ , similar to the  $V_{max}$  determined for the DmGCL holoenzyme (8.45  $\mu\text{mol}/\text{min}/\text{mg}$ ) generated from the same preparation of DmGCLC. These results indicate that HsGCLM is able to modify the activity of DmGCLC in a similar way to DmGCLM.

**Effect of DmGCLM Ablation on Glutathione Homeostasis in Vivo**—The gene encoding DmGCLM (*Gclm*) maps to 94C on the third chromosome. We obtained the P-element-induced recessive lethal mutant stock *l(3)L0580*, which contains a P[*lacW*]-element inserted in the 5'-noncoding region of the *Gclm* gene. The P-element in this stock was not responsible for the lethality, as judged from failure to recover dysgenically induced revertants, from failure to rescue lethality with a transgene, and by deficiency mapping (data not shown). Accordingly, we

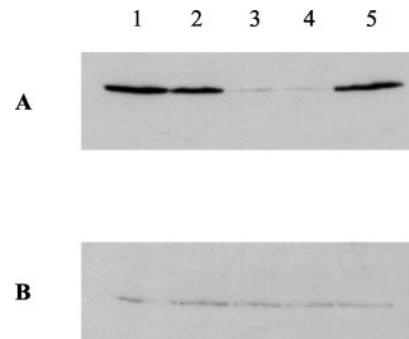


FIG. 9. **DmGCL protein levels in *Gclm*<sup>L0580</sup> flies.** Portions (5  $\mu\text{l}$  for analysis of DmGCLM or 10  $\mu\text{l}$  for analysis of DmGCLC) of fly homogenates from wild-type, mutant, or revertant strains were analyzed by Western blotting using antiserum raised against DmGCLM (A) or DmGCLC (B). Lane 1, Canton S; lane 2, *w*<sup>1118</sup>; lane 3, *e Gclm*<sup>L0580</sup>; lane 4, *Gclm*<sup>L0580 ro</sup>; lane 5, *e Gclm*<sup>rev4</sup>.

separated the recessive lethal mutation in this stock from the P-element by recombination with an *e<sup>4</sup> wo<sup>1</sup> ro<sup>1</sup>* chromosome. This yielded two fully viable recombinants bearing the P-element insertion, *e<sup>4</sup> Gclm*<sup>L0580</sup> and *Gclm*<sup>L0580 ro<sup>1</sup>. We have named this P-element allele *Gclm*<sup>L0580</sup> and refer here to the two marked recombinant derivatives as *e Gclm*<sup>L0580</sup> and *Gclm*<sup>L0580 ro</sup>. Levels of DmGCLM in homozygous *e Gclm*<sup>L0580</sup> and *Gclm*<sup>L0580 ro</sup> flies were examined by Western blotting. Fig. 9A shows that DmGCLM was substantially diminished in *e Gclm*<sup>L0580</sup> or *Gclm*<sup>L0580 ro</sup> fly lysates. Fig. 9B shows that the reduction in DmGCLM was not accompanied by any change in DmGCLC protein levels. Northern blotting showed a dramatic reduction in *Gclm* transcription in adult *Gclm*<sup>L0580</sup> homozygotes (data not shown). Collectively, these data indicate that *Gclm*<sup>L0580</sup> is a severely hypomorphic allele. *Gclm*<sup>L0580</sup> homozygous flies are viable and fertile under normal laboratory conditions and have no obvious phenotype. We obtained a revertant (*e Gclm*<sup>rev4</sup>) by mobilizing the *L0580* element from the *e Gclm*<sup>L0580</sup> chromosome. The *e Gclm*<sup>rev4</sup> homozygotes have wild-type levels of DmGCLM, as judged by Western blotting (Fig. 9).</sup>

As DmGCLM enhances the catalytic efficiency of DmGCLC and reduces its sensitivity to feedback inhibition by glutathione, we hypothesized that *e Gclm*<sup>L0580</sup> and *Gclm*<sup>L0580 ro</sup> flies may have an impaired capacity to synthesize glutathione. We analyzed the whole body glutathione contents of *e Gclm*<sup>L0580</sup> and *Gclm*<sup>L0580 ro</sup> flies and compared them with those of Canton S and *w*<sup>1118</sup> flies, both of which are wild-type for *Gclm*, and with the revertant, *e Gclm*<sup>rev4</sup> (Fig. 10). Canton S and *w*<sup>1118</sup> flies contained  $348.15 \pm 20.9$  and  $305.5 \pm 27.3$  pmol of glutathione/fly, respectively. The revertant strain (*e Gclm*<sup>rev4</sup>) contained slightly less glutathione than the wild-type strains



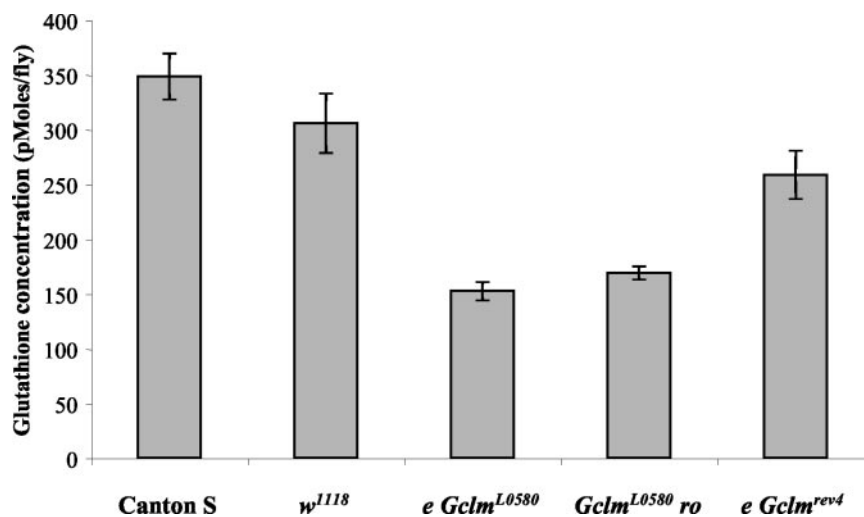


FIG. 10. Effect of mutation of *Gclm* on glutathione levels *in vivo*. Glutathione levels in whole fly homogenates were determined as described under "Experimental Procedures." Glutathione concentrations are expressed as the mean  $\pm$  S.D. of three experiments. Glutathione levels differed significantly by genotype (analysis of variance,  $F_{(4,40)} = 68.42$ ,  $p < 0.001$ ). *Post hoc* comparisons indicated that the levels in Canton S and *w<sup>1118</sup>* flies did not differ significantly from each other, but did differ from the levels in the other three genotypes ( $p < 0.001$ ); the levels in the two *Gclm<sup>L0580</sup>* stocks did not differ from each other, but did differ from the levels in the other three genotypes ( $p < 0.001$ ); and the levels of glutathione in the *Gclm<sup>rev4</sup>* stock were significantly different from those in each of the other genotypes in the analysis.

( $258.52 \pm 22$  pmol of glutathione/fly). The concentrations of glutathione in the *e Gclm<sup>L0580</sup>* and *Gclm<sup>L0580</sup> ro* flies were substantially less ( $152.47 \pm 8.35$  and  $168.89 \pm 6.01$  pmol of glutathione/fly, respectively) than those in all of the control strains, implicating DmGCLM as an important component of the regulatory pathway for glutathione synthesis *in vivo*.

#### DISCUSSION

Glutamate-cysteine ligase has a profound influence on intracellular redox status. Despite this, the complex regulatory mechanisms that modify GCL activity to control glutathione homeostasis remain poorly understood. In this study, we have examined the role of intermolecular disulfide linkages in regulating *Drosophila* GCL activity and showed that abrogation of the ability to form disulfide bridges between the catalytic and modifier subunits has a significant impact on the catalytic efficiency of the holoenzyme as well as sensitivity to feedback inhibition by glutathione. The proposed importance of DmGCLM in regulating glutathione homeostasis *in vivo* was substantiated by the observation that *Drosophila* strains with a mutation in *Gclm* have approximately half as much glutathione as wild-type strains.

Using a combination of MALDI-TOF mass spectroscopy and examination of evolutionary conservation of cysteines, we identified Cys<sup>213</sup>, Cys<sup>214</sup>, and Cys<sup>267</sup> in DmGCLM as candidate disulfide-forming cysteines important for the interactions with DmGCLC. A mutant form of DmGCLM that lacks these 3 cysteine residues (DmGCLM-ABD) could form a stable holoenzyme complex with DmGCLC, but was unable to form intermolecular disulfide bridges under non-reducing conditions. Our kinetic analyses showed that the mutant DmGCLC/DmGCLM-ABD holoenzyme was less active than the unmodified holoenzyme, but significantly more active than DmGCLC, supporting the notion that both covalent and non-covalent interactions are important in regulating DmGCL activity. We also found that the DmGCLC/DmGCLM-ABD mutant was more sensitive to inhibition by glutathione than the wild-type holoenzyme. This observation is in keeping with the hypothesis that disulfide linkages generate a conformational change that causes the active site to be less susceptible to competitive inhibition by glutathione (9); in the absence of disulfide bridges (or where intermolecular disulfides are reduced by glutathione), the active site would adopt a more open conformation, which would

allow access of glutathione and competition with glutamate. Interestingly, the degree of inhibition observed for DmGCLC/DmGCLM-ABD in the presence of very high concentrations of glutathione was still greater than that observed for wild-type DmGCL. It would be expected that, under such conditions, the disulfide linkages between DmGCLC and DmGCLM would be fully reduced, and the sensitivity of wild-type DmGCL to glutathione inhibition would be identical to that of the DmGCLC/DmGCLM-ABD mutant. It is possible, however, that a proportion of the recombinant holoenzyme contains glutathione-resistant disulfides. Pertinently, it was shown previously that a proportion of native rat kidney GCL remained as undissociated holoenzyme when subjected to SDS-PAGE after incubation with the physiologically attainable concentration of 10 mM glutathione (9).

At the outset of this study, we noticed that replacing the N-terminal histidine tag with one at the C terminus caused GCL activity to increase by >10-fold. This implies that the N-terminal tag may interfere with the conformation of the protein. Although we have not analyzed DmGCLC activity without a histidine tag, previous studies have shown that an N-terminal histidine tag has a modest inhibitory effect on HsGCLC activity (11). The low activity of N-terminally tagged DmGCLC made it less amenable to study than the more active C-terminally tagged protein, and therefore, we carried out all of our subunit interaction studies using the latter DmGCLC form. Furthermore, to circumvent potential problems due to the rapid oxidation of the cysteine substrate *in vitro*, we performed our enzyme assays with  $\alpha$ -aminobutyrate rather than cysteine, which we had used previously (13). We were surprised, however, to find that there was no significant difference in the  $K_m$  values for glutamate between DmGCLC and either the mutant or wild-type DmGCL holoenzyme. In our earlier study (using cysteine as substrate and DmGCLC with an N-terminal tag), we found that the  $K_m$  value for glutamate was nearly six times higher when the modifier subunit was absent. This difference in  $K_m$  values is highly reproducible. We are uncertain why the more active C-terminally tagged DmGCLC does not exhibit this DmGCLM-dependent difference in  $K_m$  for glutamate. It is possible that the cysteine residue that has been proposed to be at or near the active site (12, 22) is more susceptible to oxidation by the cysteine substrate in uncomplexed DmGCLC due to

DmGCLM-induced conformational alterations. Indeed, we have shown previously that DmGCLC is more susceptible to inactivation by cystamine than the DmGCLC holoenzyme (13). Alternatively, it is possible that oxidation of cysteine to cystine in the assay mixture could cause differential inhibition, which might be reflected in apparent differences in  $K_m$  values. One other possibility is that the C-terminal histidine tag on DmGCLC actually generates a conformational change in the catalytic subunit to increase activity and to decrease the  $K_m$  for glutamate. Despite this caveat, we have demonstrated convincingly that interaction with DmGCLM increases the catalytic efficiency of DmGCLC and that the potential to form intersubunit disulfide linkages can further modulate activity and susceptibility to inhibition by glutathione.

We found that HsGCLM was able to functionally substitute for DmGCLM in the regulation of DmGCLC activity. The modifying effect of HsGCLM was very similar to that of unmodified DmGCLM, suggesting that intersubunit disulfide bridges may form between HsGCLM and DmGCLC to enhance activity. Supporting this notion, HsGCLM was able to form disulfide bonds with DmGCLC. These data strongly implicate Cys<sup>213</sup> and/or Cys<sup>214</sup> as the principal modulator of GCLC activity, as Cys<sup>213</sup> and Cys<sup>214</sup> are the only cysteines that are conserved between HsGCLM and DmGCLM. We should note, however, that mutant DmGCLM polypeptides in which only Cys<sup>213</sup> and Cys<sup>214</sup> were changed to serine could still form covalent linkages with DmGCLC when analyzed by SDS-PAGE under non-reducing conditions (data not shown). In this study, it was therefore important to perform the biochemical analyses on GCL protein complexes in which the formation of disulfide linkages *in vitro* was not apparent.

In view of the number of hypothetical GCLM proteins identified from representatives of a variety of eukaryotic classes, it seems likely that many eukaryotes utilize a modifier subunit to regulate glutathione synthesis. Nevertheless, it probable that this mechanism is not common to all eukaryotes. Previous work has shown that GCL from *Trypanosoma brucei* is highly unlikely to be regulated by a modifier subunit (23). This hypothesis is substantiated by the fact that *T. brucei* GCL lacks the conserved cysteine residue (Cys<sup>553</sup> in HsGCLC) that has been proposed to be involved in disulfide interactions between HsGCLC and HsGCLM (11). When we examined GCLC sequences from organisms in which we found a hypothetical GCLM subunit (*D. rerio*, *X. laevis*, *G. gallus*, *C. elegans*, *Anopheles gambiae*, *S. pombe*, and *Neurospora crassa*), as well as the characterized mammalian forms, all were found to contain the conserved Cys<sup>553</sup>, supporting the hypothesis that this cysteine residue plays an important role in regulating GCL activity by participating in intermolecular disulfide bridge formation. It is of interest to note that, unlike *S. pombe* GCLC, *Saccharomyces cerevisiae* GCLC does not contain the conserved Cys<sup>553</sup>, and we were unable to find a GCLM ortholog in the genome sequence.

Despite the wide distribution of GCLM, *Gclm*<sup>L0580</sup> flies are fully viable and fertile. The P-element insertion in the 5'-untranslated region of *Gclm* almost entirely ablates expres-

sion, and the mutants have ~50% less glutathione than control strains. However, the lack of phenotype under standard laboratory conditions is perhaps not surprising in light of a recent study in which targeted disruption of mouse *Gclm* caused no overt phenotype, despite causing significant decreases in glutathione levels in all tissues examined (24). *Gclm*<sup>-/-</sup> mouse fetal fibroblasts were, however, substantially more susceptible to H<sub>2</sub>O<sub>2</sub> toxicity than those with the *Gclm*<sup>+/-</sup> or *Gclm*<sup>+/+</sup> genotype.

The genetic experiments with fruit flies and mice have highlighted the importance of GCLM in glutathione homeostasis *in vivo*. It is likely that an impaired capacity to synthesize glutathione would sensitize mutant *Gclm* animals to cellular oxidative damage. As oxidative stress has been proposed to play a prominent role in aging (25), it will be interesting to study the relationship between glutathione homeostasis, resistance to various stresses, and aging in mutant *Gclm* flies. The use of *Drosophila* as a model system will allow us to address the importance of reversible disulfide bridges as a mechanism for regulating glutathione synthesis *in vivo*. We should be able to use gene replacement strategies to generate flies that express the DmGCLM-ABD mutant instead of the wild-type protein, furthering our understanding of the fundamental mechanisms of regulation and the biological relevance of this essential thiol antioxidant.

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**The Modifier Subunit of *Drosophila* Glutamate-Cysteine Ligase Regulates Catalytic Activity by Covalent and Noncovalent Interactions and Influences Glutathione Homeostasis *in Vivo***

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